



Concentration of biologically active compounds extracted from *Ilex paraguariensis* St. Hil. by nanofiltration



Aureanna Nairne Negrão Murakami^{a,*}, Renata Dias de Mello Castanho Amboni^{a,*},
Elane Schwinden Prudêncio^a, Edna Regina Amante^a, Carlise Beddin Fritzen-Freire^a,
Brunna Cristina Bremer Boaventura^a, Isabella de Bona Muñoz^a, Catia dos Santos Branco^b,
Miriam Salvador^b, Marcelo Maraschin^c

^a Department of Food Science and Technology, Federal University of Santa Catarina, Rodovia Admar Gonzaga, 1346, 88034-001 Florianópolis, SC, Brazil

^b Department of Biochemistry, University of Caxias do Sul, 95070-560 Caxias do Sul, RS, Brazil

^c Department of Phytotechny, Federal University of Santa Catarina, Rodovia Admar Gonzaga, 1346, 88034-001 Florianópolis, SC, Brazil

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ABSTRACT

The aim of this study was to characterise the bioactive compounds in mate (*Ilex paraguariensis* St. Hil) extract and in concentrated mate extract obtained by nanofiltration (NF). Also, the impact of NF on the antioxidant activity of both mate extracts was evaluated *in vitro* and using eukaryotic cells of *Saccharomyces cerevisiae* (yeast assay). The results showed a significant increase in the contents of total phenolics (338%), chlorogenic acid (483%), theobromine (323%), caffeine (251%), chlorophyll (321%), condensed tannins (278%) and saponins (211%) in the concentrated mate extract. The concentrated mate extract showed higher *in vitro* antioxidant activity than the mate extract. According to the results obtained, it can be stated that the use of nanofiltration membrane is a valid approach for the concentration of biologically active compounds in aqueous extract of mate.

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1. Introduction

The *Ilex paraguariensis* St. Hil., more commonly known as yerba-mate or mate, is a plant that is native to the subtropical region of South America and is widely consumed and produced in southern Brazil, Argentina, Uruguay and Paraguay. In these countries, the infusion or decoction of the aerial parts of mate is widely used to make different beverages such as *chimarrão* and *tererê*, which are consumed in amounts of more than one litre per day by millions of people and are also the main alternative to coffee and tea (Morais et al., 2009). This plant is very rich in different biologically active compounds, such as phenols, methylxanthines, triterpene saponins, flavonoids, minerals, and others. It is widely used in folk medicine because of its many health-promoting effects, such as anti-inflammatory, anti-obesity and anti-cancer, and mainly antioxidant activity (Heck & Mejia, 2007).

* Corresponding author. Address: Laboratório de Frutas e Hortaliças, Departamento de Ciência e Tecnologia de Alimentos, Centro de Ciências Agrárias, Universidade Federal de Santa Catarina, Rodovia Admar Gonzaga, 1346 Itacorubi, 88034-001 Florianópolis, SC, Brazil. Tel.: +55 48 37215384; fax: +55 48 37219943.
E-mail address: ramboni@cca.ufsc.br (Renata Dias de Mello Castanho Amboni).

Concentration of the biologically active compounds present in mate is generally performed by solid–liquid extraction, which promotes a significant dilution. The occurrence of such dilution is attributable to several factors, such as limited quantity of solid content and overall nutritional composition, which can vary according to the different regions and times of harvesting, among other factors. Besides, the traditional approaches used for concentrating biologically active compounds from natural products include simple steam-and-vacuum distillation, which generally requires high temperature and high energy consumption. These methods may result in nutritional loss caused by the instability of bioactive compounds, due to the application of a high temperature for a long period of time (Sonaglio, Ortega, Petrovick, & Bassani, 2007).

The utilisation of membrane technologies for concentrating bioactive compounds from natural products has been successfully employed, for example, with *Ginkgo biloba* extract (Xu & Wang, 2005). Compared to the traditional methods used for concentrating biologically active compounds, membrane concentration process reveals new possibilities because of advantages, such as working at ordinary temperatures, absence of phase transition, and low energy consumption (Santamaría, Salazar, Beltrán, & Cabezas, 2002). This procedure is based on the principle of selective perme-

ation of the solute molecules through semi-permeable membranes. The liquid that is retained by the membrane is called concentrate and the liquid that passes through it is called permeate. In most membrane processes, such as microfiltration, ultrafiltration, nanofiltration, and reverse osmosis, the driving force for mass transfer across the membrane is mechanical pressure (Maroulis & Saravacos, 2003, chap. 10). The main advantage of employing NF membranes for the concentration of bioactive compounds of mate is that by selecting membranes with suitable molecular weight cut-off (MWCO), this technology can be used to fractionate molecules of similar molecular weight (100–1000 Da range).

The aim of this work was to characterise the bioactive compounds in extract and concentrated extract of *Ilex paraguariensis* St. Hil. Besides evaluating the effects of NF on these valuable bioactive compounds, in this work we also evaluated the antioxidant activity of these mate extracts *in vitro* and using eukaryotic cells of *Saccharomyces cerevisiae* (yeast assay).

2. Material and methods

2.1. Chemicals

Hydrochloric acid, sodium hydroxide, sodium carbonate, ethanol, methanol, dichloromethane, chloroform, acetonitrile, glacial acetic acid, sodium chloride, formic acid and *n*-butanol were obtained from Vetec (Duque de Caxias, RJ, Brazil). The Folin–Ciocalteu phenol reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), vanillin, tannic acid, chlorogenic acid, gallic acid, perchloric acid, caffeine, theobromine, ursolic acid and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO). All reagents were either of analytical or of chromatographic grade.

2.2. Plant material

The leaves of *Ilex paraguariensis* St. Hil. used for the preparation of the extract were harvested in Catanduvas, Santa Catarina state, Brazil. After the leaves were picked, they were washed and the excess water on the surface of the leaves was removed by centrifugation. After that, the leaves were dried in a forced air oven (FABBE, 171, São Paulo, Brazil) for 24 h at 45 °C and then they were ground with a knife mill (Marconi, MA-580, Piracicaba, Brazil) until reaching a particle size smaller than 3.55 mm. The ground leaves were placed in plastic bags and kept at –18 °C until the preparation of the aqueous extracts.

2.3. Preparation of the mate extract

The mate extract was produced according to Murakami et al. (2011), using 3 g of ground leaves suspended in 100 mL of distilled water with constant stirring. The pH was adjusted to 6.0 by adding either 0.1 N hydrochloric acid or 0.1 N sodium hydroxide solutions and using a pH meter (Quimis, Q-400A, Diadema, SP, Brazil). The extracts were filtered with filter paper (12.5 cm diameter and 25 µm pore size) using a vacuum pump (Primatec, 131, Itu, Brazil) and the extract volumes were made up to 100 mL with distilled water (referred to as 3 g/100 mL extracts).

2.4. Concentration of the mate extract

Nanofiltration (NF) was applied to concentrate biologically valuable components of mate extract. The concentration was performed with a tangential filtration system on a pilot scale equipped with a spiral membrane module (Osmonics membranes, HL2521TF, Minnetonka, MN), with a MWCO ranging between

150 and 300 Da, 0.6 m² of filtration area and 98% rejection of MgSO₄ in a test performed by manufacturing with a spiral module at 25 °C and 690 kPa. The experiment was performed on pilot equipment (Fig. 1) that allows batch circulation, meaning that both the permeate and the concentrate could be carried back to the feed tank. The permeate was totally removed, in order to obtain the concentrated mate extract.

The operating parameters during the NF process were temperature of 24 ± 1 °C and pressure of 300 kPa, up to a VRF (volumetric reduction factor) of 4. As was expected, a decrease in permeate flux was observed throughout the time, due to concentration polarisation and fouling of membrane. A permeate flux was obtained between 0.50 and 24.0 L/h.m², while the average permeate flux was equal to 4.53 L/h.m². After each processing, the pilot unit and membrane were cleaned and sterilised, according to the manufacturer's instructions.

2.5. Analytical methods

The characterisation of the mate extract and concentrated mate extract was used to evaluate the effects of membrane separation on the profile of the biologically active compounds present in the leaves of *Ilex paraguariensis*.

2.5.1. Total phenolic content and chlorogenic acid

The total polyphenol contents (TPC) of the extracts were determined following a Folin–Ciocalteu procedure (Singleton, Joseph, & Rossi, 1965). The appropriate dilutions of extracts were oxidised with Folin–Ciocalteu reagent and its reaction was neutralised with sodium carbonate. The absorbance of the resulting blue colour was measured at 765 nm, after 60 min, with a UV–Vis spectrophotometer (Model U-1800; Hitachi, Tokyo, Japan). The TPC was expressed as gallic acid equivalents (GAE) in mg per mL of extract (mg GAE/mL).

The determination of chlorogenic acid was performed using an HPLC system (Shimadzu LC-10, Kyoto, Japan) equipped with a reverse-phase column (Shim-pack C18, 4.6 mm Ø × 250 mm), thermostated at 40 °C, and a UV–Vis detector (Shimadzu SPD 10A, λ = 280 nm). An isocratic mobile phase of water:acetic acid:*n*-butanol (350:1:10 v/v/v) was used at a flow rate of 0.8 mL/min. The injection volume was 10 µL. For the quantitative analysis, a standard calibration curve was obtained by plotting the peak area against different concentrations of chlorogenic acid. The curve showed a good linearity and followed Beer's Law ($r^2 = 0.99$). Similarly, the final concentration of chlorogenic acid present in the samples was determined as average content after three consecutive injections.

2.5.2. Methylxanthine compounds

A volume of 15 mL of each extract was treated with 60 mL of dichloromethane for 1 h and the organic phase was concentrated to 2 mL under reduced pressure. The HPLC analysis was performed on a Shimadzu LC-10A system equipped with a UV–Vis detector SPD 10A set at 272 nm. The experiments were carried out on a reversed-phase Shim-pack C18 (4.6 mm Ø × 250 mm) column. The system was operated isocratically at 30 °C using a mobile phase composed of acetonitrile:0.1% formic acid (15:85 v/v), with a flow rate of 1.0 mL/min. Prior to injection, all the samples were centrifuged at 2800g for 10 min (Hermle, Z 200A, Wehingen, Germany) and filtered through 0.22-µm micropore membranes.

The injection volume was 10 µL for the mate extract and 5 µL for the concentrated mate extract. For quantitative analysis, standard calibration curves were obtained for caffeine (1.0–100.0 µg/mL; $r^2 = 0.99$) and for theobromine (2.5–50 µg/mL; $r^2 = 0.99$). The final concentrations of each sample were determined by three consecutive injections (Strassmann et al., 2008).

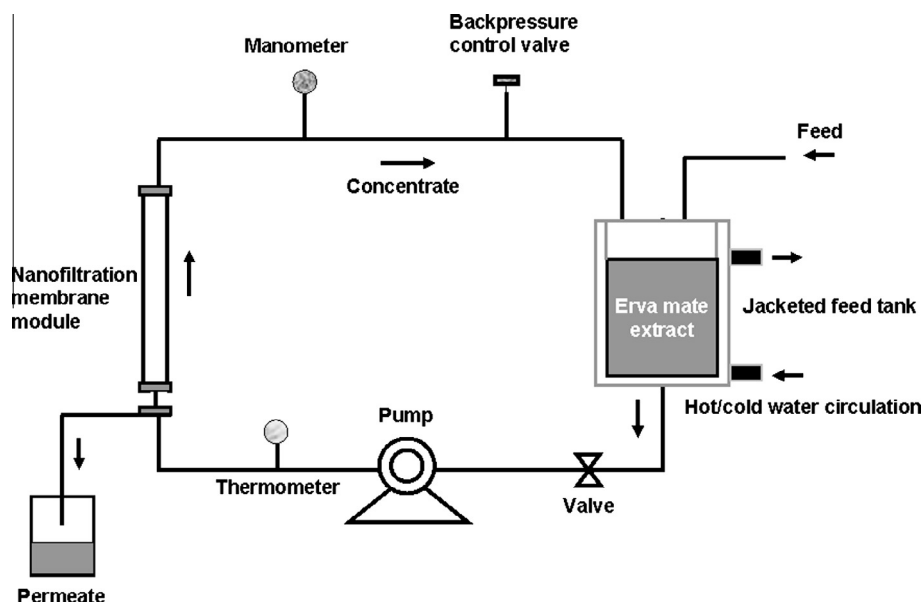


Fig. 1. Schematic diagram of the nanofiltration unit.

2.5.3. Total saponins content

The extraction procedure was performed according to Gnoatto, Shenkel, and Bassani (2005). The saponins present in 10 mL of each of the two extracts were hydrolysed with 5 mL of HCl (12 M) for 2 h under reflux. The saponins were extracted with 6 mL of chloroform. The organic phase was evaporated in a rotary evaporator and the residue was resuspended in 10 mL of ethanol.

Determination of the total triterpene saponins content was conducted using the colorimetric method of Xiang, Tang, Chen, and Shi (2001) with slight modifications. The principle of this method is the reaction of oxidised triterpene saponins with vanillin. Perchloric acid is the strong oxidant and the distinctive colour of this reaction is purple. Aliquots from the ethanolic phase were totally evaporated and then 150 μ L of 5% (w/v) vanillin/glacial acetic acid solution and 500 μ L of perchloric acid were added to the dry residue obtained. The mixture was incubated at 60 °C for 45 min, cooled down in an ice bath and then added with glacial acetic acid. The absorbance rate was measured with a UV/Vis spectrophotometer (Hitachi, U-1800) at 548 nm. The total saponins content was quantified by using a standard calibration curve of ursolic acid ($y = 0.00087205 - 0.02339x$, $r^2 = 0.99$), the major triterpenic nucleus of *Ilex paraguariensis*.

2.5.4. Condensed tannins content

The methodology to determine the condensed tannins content is described in this present work consists of two stages: stage A, where the total polyphenols are quantified; and stage B, where residual polyphenols are quantified after adsorption of tannins by gelatin (Valdes, Leyes, & León, 2000). In stage A, 4.5 mL of extract were treated by adding 1 mL of Folin–Ciocalteu reagent and 0.5 mL of sodium carbonate (20%). This mixture was then stirred and the volume was made up with distilled water to 125 mL. The absorbance was measured with a UV/Vis spectrophotometer (Hitachi, U-1800) at 700 nm, after 2 min. In stage B, 10 mL of extract were diluted in 40 mL of distilled water and added with 25 mL of gelatin (25%). After that, 50 mL of an acidified saturated solution of 1% sodium chloride and 5 g of kaolin were added to the solution. The solution was then capped and stirred for 30 min (700g) and immediately afterwards it was filtered. From this solution, the procedure of stage A was repeated. The condensed tannins were quantified by using a standard calibration curve of tannic acid (1.0–10.0 mg/mL; $r^2 = 0.97$; $y = 0.0869x + 0.0785$).

2.5.5. Chlorophyll content

One millilitre of each extract was added to 7 mL of dimethyl sulfoxide (DMSO) and the mixture was then placed in an oven at 65 °C for 15–20 min. After that, 3 mL of the obtained solution were analysed with a UV/Vis spectrophotometer (Hitachi U-1800) set at 645 and 663 nm. The DMSO reagent was used as a blank solution. The results showed the content of total chlorophyll (Hiscox & Israelstam, 1979).

2.5.6. Antioxidant activity in vitro

The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay was based on the method proposed by Brand-Williams, Cuvelier, and Berset (1995). An aliquot of 0.1 mL of each extract was mixed with 3.9 mL DPPH in methanol (60 μ M). The mixture was vigorously shaken and then the absorbance rate was measured at 515 nm every 10 min until it stabilised (Hitachi U-1800). Methanol was used as a blank instead of DPPH solution. EC_{50} value (μ g extract/mL) is the effective concentration at which DPPH radicals were scavenged by 50% and it was obtained by interpolation from linear regression analysis.

2.5.7. Antioxidant activity (yeast assay)

The evaluation of antioxidant activity was carried out using eukaryotic cells of *S. cerevisiae* strain XV185-14c (MAT α , ade2-2, arg4-17, his1-7, lys1-1, trp5-48, hom3-10). Yeast cell suspensions containing 2×10^7 cells/mL (exponential phase) were treated with 75 mM hydrogen peroxide, in the presence of concentrated and mate extracts. This dilution was the highest non-cytotoxic concentration of both extracts determined in preliminary assays. The tubes were incubated for 1 h at 28 °C. The samples were then serially diluted in saline solution (0.9% w/v), plated onto YPD (10 g/L of yeast extract, 20 g/L of peptone, 20 g/L of dextrose and 20 g/L of agar-agar) and incubated at 28 °C for 48 h. After incubation, the colonies were counted, and 100% survival was considered the total number of colonies observed on the control plate (untreated cells) (Wilmsen, Spada, & Salvador, 2005).

2.6. Statistical analysis

All the data were evaluated using the software STATISTICA version 6.0 (2001) (StatSoft Inc., Tulsa, OK) and expressed as mean \pm standard deviation (SD) of triplicate measurements. Tu-

Table 1

Chemical compositions of the mate extract and of the concentrated mate extract.

	Concentrated mate extract	Mate extract
Total phenolic content (mg GAE/mL)	7.82 ± 0.039 ^a	2.32 ± 0.003 ^b
Chlorogenic acid (μg/mL)	9004 ± 165 ^a	1862 ± 3.49 ^b
Methylxanthines (μg/mL)		
Caffeine	3142 ± 367 ^a	1253 ± 72.5 ^b
Theobromine	171 ± 10.51 ^a	53.1 ± 1.44 ^b
Total saponins content (μg/mL)	366 ± 14.01 ^a	173 ± 5.14 ^b
Condensed tannins content (g TA/100 g d.e.)	35.6 ± 0.42 ^a	12.8 ± 0.74 ^b
Total chlorophyll content (μg/mL)	36.0 ± 3.42 ^a	11.2 ± 0.31 ^b

Data are mean ± SD (n = 3); TA: Tannic acid; d.e.: dry extract.

Mean values in the same line followed by different letters are significantly different (p < 0.05).

key's studentised range test was carried out to test for any significant differences between the mate extract and concentrated mate extract. A difference was considered statistically significant when $p < 0.05$.

3. Results and discussion

The chemical compositions of the mate extract and the concentrated mate extract are shown in Table 1. Significant changes occur in the concentration of bioactive compounds of mate extract after nanofiltration (NF). Concentration by NF does not occur only because of the MWCO of the membrane; it can also occur because of the common structural properties of hydrophobic compounds, which generally include aromatic (benzene) ring structures that have aliphatic carbon groups which, if undissociated in pH conditions lower than their pK_a values, can be readily adsorbed in this kind of hydrophobic membrane (Yoon, Westerhoff, Snyder, & Wert, 2006).

As noted by Murakami et al. (2011) and by Prudêncio et al. (2012) with mate leaf extract and mate bark extract, in this present study it was possible to concentrate bioactive compounds from mate extract by NF (Table 1). The content of chlorogenic acid was determined because it is present in higher concentrations in mate (Pagliosa et al., 2010). Fig. 2(a) shows a representative HPLC-DAD chromatogram of chlorogenic acid identified and quantified in the mate samples. The same behaviour obtained by Murakami et al. (2011) was verified in this present study, i.e., the concentration of chlorogenic acid increased after nanofiltration.

The methylxanthine content in the concentrated mate extract was higher ($p < 0.05$) than in the mate extract. Fig. 2(b) shows a representative HPLC-DAD chromatogram of the methylxanthines identified and quantified in the mate samples. After nanofiltration, the concentration of theobromine (323%) increased more than the concentration of caffeine (251%). As expected, the theobromine content was much lower than the caffeine content in both extracts. The large amount of caffeine in the leaves of *Ilex paraguariensis* not only is responsible for the stimulant effect of mate, but it is also responsible for several other biological activities. Silva et al. (2011) suggested that mate consumption could decrease body weight and adiposity, most probably because of caffeine's ability to increase thermogenesis. Moreover, Strassmann et al. (2008) noted that treatments performed with mate extract and with caffeine on the vascular membranes of chick embryos yolk sac showed pro-vasculogenic and angiogenic properties and also embryonic growth enhancement. However, based on available evidence, it is suggested that caffeine intake should be less than 300 mg caffeine per day (equivalent to 4.6 mg/kg/day in a 65 kg person) while children should consume less than 2.5 mg/kg/day; Nawrot et al., 2003).

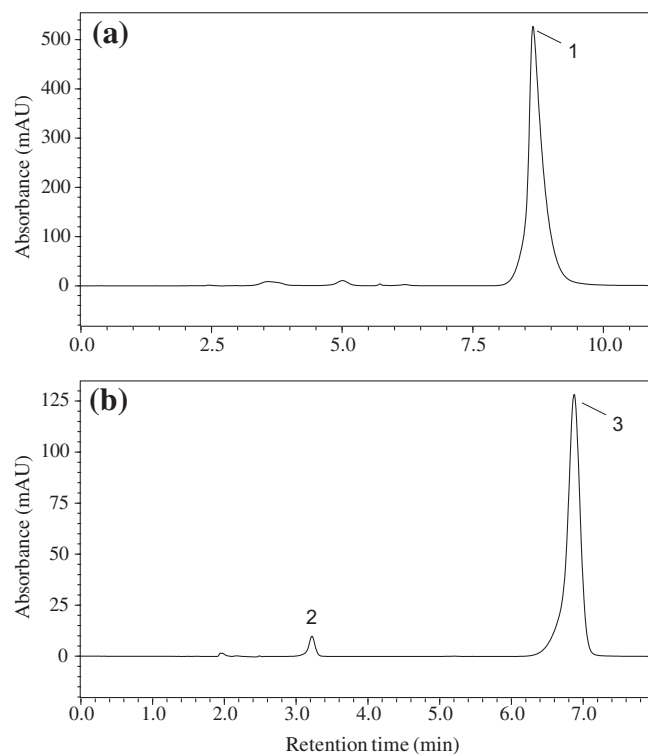


Fig. 2. Representative HPLC-DAD chromatogram of mate extract: (a) phenolic compounds with signal at 280 nm; and (b) methylxanthines with signal at 272 nm; (1) chlorogenic acid; (2) theobromine; (3) caffeine.

The leaves of *Ilex paraguariensis* contain a significant amount of triterpenoid saponins. These compounds are highly water-soluble and are responsible for the typically bitter taste of mate. Saponins may be used as a chemical fingerprint for authentication of mate. Adulteration by variable quantities of leaves of other *Ilex* species is rather common (Heck & Mejia, 2007). Concentration of mate extract by NF increased the total saponin content by 211%. According to the data in Table 1, the total saponin content of the concentrated mate extract was 366 ± 14.0 μg/mL. Similar results were reported by Silva et al. (2011) for aqueous extract of unprocessed mate, 387 ± 1.58 μg/mL; however, the concentration of the extract used in that study was 7 g of ground leaves in 100 mL of water. Such high saponin content in concentrated mate extract not only affects the flavour of the extract, but also provides hypocholesterolaemic properties. The potential cholesterol-lowering properties were reported in a work of Morais et al. (2009), where consumption of approximately 130 and 350 mg of saponins in yerba mate infusion provided further decrease in LDL-cholesterol in subjects undergoing statin therapy. Puangphaphant, Berhow, and Mejia (2011) also stated that the saponins of mate prevent inflammation and colon cancer *in vitro*.

The concentrated mate extract showed an amount of condensed tannins approximately 278% higher than the mate extract. Studies on condensed tannins take on greater importance when considering the impact of these compounds on nutrition. Despite the known antinutritional activity of condensed tannins, Okuda (2005) reported several health-promoting effects of these compounds, such as inhibition of lipid peroxidation, carcinogen mutagenicity, and tumours, and at the same time they promote antiviral activity, and potentiation of antibacterial activity.

In addition, according to the results shown in Table 1, the concentration of total chlorophyll content in the concentrated mate extract obtained by NF was 321% higher compared to that of the mate extract. Ferruzzi and Blakeslee (2007) reported that chloro-

phylls have properties that are proven to benefit the human body, such as antioxidant activity, antimutagenic activity, modulation of xenobiotic metabolising enzymes, and induction of apoptotic events in cancer cell lines. Moreover, chlorophyll gives an indirect estimation of the nutrient status because much of the leaf nitrogen is incorporated in chlorophyll (Ferruzzi & Blakeslee, 2007).

Antioxidants are substances that counteract free radicals and prevent the damage they cause. These substances can greatly reduce the damage caused by oxidants by breaking them down before they react with biological targets, preventing either chain reactions or activation of oxygen to give highly reactive products (Ratnam, Ankola, Bhardwaj, Sahana, & Ravi Kumar, 2006). The results in Table 2 show that the concentrated mate extract had better ($p < 0.05$) antioxidant activity *in vitro* in comparison to the mate extract. Prudêncio et al. (2012) and Boaventura et al. (2012) also noted substantial differences between DPPH EC₅₀ in the mate aqueous extracts of bark and leaves after concentration processes. The well-known antioxidant activity of *Ilex paraguariensis* is related to the presence of several compounds, mainly chlorogenic acid (Anesini, Turner, Cogoi, & Filip, 2012). However, tests that measure antioxidant activity *in vitro* do not always show this behaviour *in vivo*. Wilmsen et al. (2005) reported that the evaluation of antioxidant activity using *S. cerevisiae* yeast as a biological system model is a quick test that shows representative results for studies of oxidative stress in eukaryotic cells treated with hydrogen peroxide.

Both the samples that were evaluated were able to protect the yeast cells against damage caused by hydrogen peroxide (Table 2). Additionally, the results obtained in this present study indicate that the phenolic compounds present in the samples play an important role in antioxidant activity. Differences ($p < 0.05$) in the survival rates of the *S. cerevisiae* yeast treated with hydrogen peroxide of mate and concentrated mate extracts were noted in this present study. However, antioxidant activity does not seem to depend exclusively on total polyphenol content. As observed by Stefenon et al. (2010) in red sparkling wines, samples with significant amounts of phenolic compounds did not show the highest antioxidant activity. Medina et al. (2011) also noted there was no correlation with the antioxidant activity measured by DPPH in araçá extract. It is possible that the lower protection of yeast cells against damage caused by hydrogen peroxide is due to condensation reactions influenced by nanofiltration. Halliwell (2008) reported that, under certain experimental conditions and in high concentrations, some polyphenols such as phenolic acids can exert pro-oxidant activities, forming compounds that have toxic effects and may cause cell death. The high concentrations of phenolic compounds in the concentrated mate extract obtained by NF probably explain their lower protective effect over yeast cells. Addi-

tional studies are under way to identify other compounds that are possibly present in these extracts and that may be further developed for nutraceutical and therapeutic applications.

4. Conclusions

It can be stated that the use of nanofiltration is a valid approach for the concentration of biologically active compounds in aqueous extract of mate. The results showed that there was a significant increase in the contents of total phenolics, chlorogenic acid, methylxanthines, chlorophyll, and saponins, all of which are compounds that may have an important role in maintaining good health. Moreover, the mate extract and the concentrated mate extract showed differences in the survival rates of the *S. cerevisiae* yeast treated with hydrogen peroxide.

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Table 2

DPPH[•] mean values for the concentrated mate extracts and mate extracts analysed, and mean survival values of *Saccharomyces cerevisiae* yeast treated with hydrogen peroxide (H₂O₂, 75 mM) in presence and absence of concentrated mate extracts and mate extracts.

	DPPH [•] EC ₅₀ value (μg/mL) ± SD
Mate extract	130 ± 4.12 ^a
Concentrated extract	4.19 ± 1.23 ^b
	Survival (%) ± SD
Water	100.00 ± 0.00 ^a
H ₂ O ₂ 75 mM	45.00 ± 0.00 ^d
Mate extract 0.01% + H ₂ O ₂ 75 mM	86.65 ± 5.65 ^b
Concentrated extract 0.01% + H ₂ O ₂ 75 mM	67.53 ± 3.53 ^c

Data are mean ± SD (standard deviation) (n = 3).

Mean values in the same line followed by different letters are significantly different ($p < 0.05$).

EC₅₀: (% of amount of samples necessary to scavenge 50% of DPPH[•]).

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